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(54) AGENT FOR SUPPRESSING TUMOUR GROWTH

(57) The invention pertains to biology and medicine, specifically, to the suppression of malignant tumour growth. The problem addressed by the invention is that of finding more effective and less toxic agents for suppressing tumour growth. The invention in essence proposes the use of a substance consisting of a cobalt or iron complex with substituted phthalocyanines (I) or naphthalocyanines (II) and of a biogenic reducing agent. In the formulae shown, R = COONa, SO_3Na, $CH_2C_5H_4N^{\oplus}Cl^{\ominus}$, $CH_2(NH_2)_2S^{\oplus}CH_2Cl^{\ominus}$. Use of the proposed agent facilitates effective suppression of a wide range of malignant neoplasms, specifically, suppression of malignant cell proliferation (*in vitro*), slowing of tumour growth in mice (*in vivo*), and a significant increase in the life expectancy of the mice by comparison with *inter alia* the use of a prototype.

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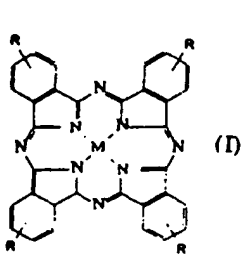

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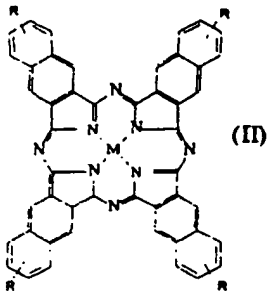


(II)

Description

The invention relates to the field of biology and medicine, in particular to suppression of the growth of malignant tumors.

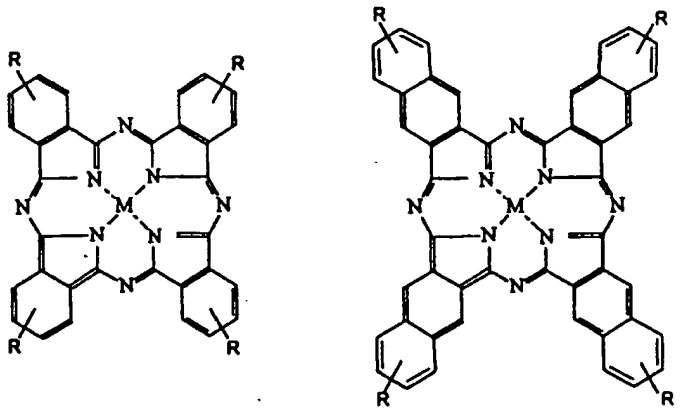
The following drugs are known for suppression of tumor growth.

1. Cis-dichlorodiaminoplatinum. Having a wide range of action, it is used for treatment of solid tumors of various localization [1, 2], but exhibits high nephrotoxicity [3].
2. Photodynamic therapy of tumors, in which photosensitizers —free tetrapyrrolic macrocyclic ligands or their complexes with metals, e.g. aluminum complex of sulfonated phthalocyanine $Al[Pc(SO_3H)_4]$ — are used. Their use is only possible in the case of surface located tumors, more exactly - accessible for a laser probe [4].
3. A drug consisting of a complex of copper (II) with glycylglycylhistidine tripeptide ([Cu(GGH)]Cl) and sodium salt of ascorbic acid in a ratio of 1:10 is the drug most similar to that proposed; it causes an increase in the life-span (ILS) of mice with Ehrlich ascitic carcinoma [5].

Drawbacks of the prototype are low effectiveness related to the low selectivity of that complex in respect of tumors and its instability in physiological conditions, and a high toxicity due to the products of decomposition of the complex.

The object of the present invention is to find more effective and less toxic drugs for suppression of tumor growth.

The essence of the proposed invention is that a drug consisting of a biogenic reductant and a complex of cobalt or iron with substituted phthalocyanines (I) or naphthalocyanines (II) is used to suppress tumor growth.



I II

where R = COONa, SO_3Na, $CH_2C_5H_4N^{\oplus}Cl^{\ominus}$, $CH_2(NH_2)_2S^{\oplus}\ C\ H_2Cl^{\ominus}$

The scientific foundation for the invention is literary data on the selective accumulation of tetrapyrrolic macrocyclic compounds and their complexes with metals in tumorous tissues [4, 6] and the facts that we have established which show the high catalytic activity of phthalocyanine complexes of cobalt and iron in model chemical systems, in particular:

1) they are homogenic catalysts of the autooxidation row of biogenic reductants and analogues thereof, e.g. ascorbic acid, ubiquinone and cysteine;
2) the (intermediate) formation of active forms of oxygen - the anion-radical of superoxide, hydrogen peroxide and a hydroxyl radical, the cytostatic and other biological activity of which is well known - takes place in this reaction [7];
3) in the conditions of this reaction, the proposed complexes cause an oxidative degradation of nucleic acids.

METHODS AND RESULTS OF TESTS

Tests of the proposed drug for cytotoxic and antitumor activity were carried out on cultures of tumor cells (in vitro) and on mice with regrafted tumors (in vivo).

Determination of the activity of the drugs on cultures of tumor cells (in vitro)

Method 1.

The method of evaluation of the cytostatic effect of a combination of phthalocyanines with ascorbic acid in a system in vitro was developed on a culture of tumor cells of human testicular carcinoma (Cao V line).

The culture of cells was grown in a monolayer in medium 199 comprising a 10% solution of an embryonic calf serum.

At the beginning of the experiment the cells were inoculated at a density of 100000/ml in a total volume of 2 ml and incubated at 37°C for 24 hours. Then the testing was carried out in the following variants:

1. Control.

The growth medium for the samples was replaced with a fresh intact nutrient medium and incubated for 48 hours, then ^{3}H-thymidine (^{3}H-T) in a final concentration of 1 μCurie/ml was introduced into the medium of the samples, it was washed with a Hank's solution, a 2.5% solution of perchloric acid, and the acid-insoluble fraction was hydrolyzed in 5 ml of 5% perchloric acid. The hydrolyzates in a volume of 100 μl were transferred into flasks with a scintillation fluid SF-8 and the level of radioactivity in the samples was registered on a RackBeta (Sweden) fluid scintillation counter. The average values of the level of radioactivity were calculated.

2. In order to evaluate the effect of ascorbic acid on the growth of cells of the Cao V line, the growth medium of the samples was replaced with a fresh medium, comprising ascorbic acid in a concentration of $1x10^{-4}$ M, incubated for 4 hours, and then the sample was processed according to the method described in para 1.

3. In order to evaluate the effect of phthalocyanine metal complexes on the growth of cells of the Cao V line, the growth medium of the samples was replaced with a fresh medium, comprising complexes in a predetermined concentration, the method described in para 1 was followed.

4. In order to evaluate the cytostatic effect of the combination of phthalocyanine and ascorbic acid, ascorbic acid was added into the growth medium comprising the complex in a ratio of concentration equal to 1:10, and the method described in para 1 was followed.

The suppression of the ^{3}H-T inclusion in the test samples was calculated according to the equation

$$\left(1 - \frac{\text{average value of decomposition/min/test samples}}{\text{average value of decomposition/min/control samples}}\right) \times 100\%$$

Statistic processing of the results was carried out using the method of sample analysis.

Method 2.

Determination of the cytostatic activity of the drugs being tested was carried out using a biotest based on inhibiting the proliferation of a regrafted culture of cells of human lung adenocarcinoma A-549, due to the action of cytotoxic agents.

In order to cultivate cells of the A-549 line, the Eagle medium was used with the addition of 100 μg/ml of heptamycin and a 10% solution of embryonic calf serum, preliminarily inactivated by heating. Cultivation of the cells was carried out under standard conditions: at 37°C in a humid atmosphere, comprising 5% carbon dioxide.

When a cytostatic biotest was being set up, 100 μl of a suspension of cells of the A-549 line at a concentration of $7.5x10^4$ cells/ml were placed in each of the craters of a flat 96-crater microboard ("Sarstedt," USA) with addition of the drugs being tested in a volume of 100 μl/crater at the beginning of the phase of logarithmic growth.

The cytostatic activity of the drugs being tested was evaluated by the colorimetric method, based on the capability of mitochondrial dehydrogenases of live test-cells of restoring exogenetically introduced soluble 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazole bromide (MTT, "Sigma Chemical Co.," USA) into an insoluble crystalline formazan. Wherein, 20 μl of a solution of MTT at a concentration of 5 mg/ml were put into each crater of the 96-crater microboard, after which the cell cultures were centrifuged, the whole volume of the culture medium was removed from the craters. For solubilization of the colored reaction products (crystals of formazan), 150 μl of dimethylsulfoxide ("Sigma") were introduced into each crater of the microboard and the microboard was incubated at room temperature and continuously shaken. The results were registered upon absorption at a wavelength of 550 nm on the minireader "Dynatech" (FRG).

The level of inhibition of proliferation of the cell cultures by the drugs being tested for cytostatic activity was calculated according to the equation:

$$IP(\%) = 100\% - \frac{P_t}{P_c} \times 100\%,$$

where:

IP is the level of inhibition of the proliferation;
P_t is the level of proliferation in the test (with the drugs): absorption of the dye in test samples;
P_c is the level of proliferation in the control (without the drugs): absorption of the dye in test samples.

Data are presented in Table 1 on the absence of cytotoxic activity of ascorbic acid (AH_2) by itself and of phthalocyanine complexes relative to cells of the Cao V and MCF-7 lines at a predetermined criterion of activity CE_{50} equal to 10^{-4} M for a 48-hour period of incubation.

Table 1. Antiproliferation activity of components of the claimed drug (phthalocyanine and naphthalocyanine complexes of metals and ascorbic acid) relative to tumor cells of a human in vitro

Content	CE_{50}, M	
	Human testicular carcinoma	Breast adenocarcinoma
	CaoV*	MCF-7 line
$Co[Pc(SO_3Na)_2]$	$2.5x10^{-4}$	
$Fe[Pc(SO_3Na)_2]$	$2x10^{-4}$	
$Fe[Pc(COONa)_8]$	$0.5x10^{-4}$	
$Co[Pc(CH_2C_5H_4N^{\oplus})_6]Cl^{\ominus}_6$	$5x10^{-4}$	$1x10^{-4}$
$Co[Pc(SO_3H)_2]$	$5x10^{-4}$	$1x10^{-4}$
$Co[Pc(CH_2(NH_2)_2SCH_2Cl)_8]$	$2.5x10^{-4}$	
$Co[Nc(CH_2C_5H_4N^{\oplus})_2]Cl^{\ominus}_2$	$1x10^{-4}$	
$Co[Pc(COONa)_8]$	$5x10^{-4}$	$1x10^{-4}$
AH_2	$>1x10^{-3}$	$>1x10^{-3}$

* AH_2 - ascorbic acid

The compound was considered to be active if CE_{50} [the concentration at which a 50% suppression of inclusions ^{3}H-T in the cells (see method 1)] was $1x10^{-4}$M under these conditions of the experiment. As is evident from the presented results, the complex $Co[Nc(CH_2C_5H_5N^{\oplus})_2]Cl^{-}_2$ and the complex $Fe[Pc(COONa)_8]$ had limited activity, all the other complexes and AH_2 were inactive.

Data are grouped in Table 2 which were obtained during a study of the inhibition of the proliferation of tumor cells in vitro when phthalocyanines and ascorbic acid were used together in noncytotoxic concentrations.

Table 2. Inhibition of the proliferation of tumor cells in the presence of phthalocyanine and naphthalocyanine complexes of metals and ascorbic acid when simultaneously administered into a culture of cells

	Inhibition of proliferation (%)		
	Human testicular carcinoma CaoV*	Breast adenocarcinoma, MCF-7* line	Human lung carcinoma A-549**
$Co[Pc(SO_3Na)_2]$	0		0
$Co[Pc(SO_3Na)_2]+AH_2$	52		83
$Fe[Pc(SO_3Na)_2]$	0		
$Fe[Pc(SO_3Na)_2]+AH_2$	12		
$Fe[Pc(COONa)_8]$	50		
$Fe[Pc(COONa)_8]+AH_2$	51		
$Co[Pc(CH_2C_5H_4N^{\oplus})_6Cl^{\ominus}_6$	0	0	0
$Co[Pc(CH_2C_5H_4N^{\oplus})_6Cl^{\ominus}_{6+AH_2}$	74	50	55
$Co[Pc(SO_3H)_2]$	0	0	
$Co[Pc(SO_3H)_2]+AH_2$	76	30	
$Co[Pc(CH_2(NH_2)_2SCH_2Cl)_8]$	0		
$Co[Pc(CH_2(NH_2)_2SCH_2Cl)_8]+AH_2$	64		
$Co[Nc(CH_2C_5H_4N^{\oplus})_2]Cl^{\ominus}_2$	0		
$Co[Nc(CH_2C_5H_4N^{\oplus})_2]Cl^{\ominus}_2+AH_2$	64		
$Co[Pc(COONa)_8]$	0	0	
$Co[Pc(COONa)_8]+AH_2$	99.5	86	

* - method 1, [complex] = $5 \cdot 10^{-5}$M, $[AH_2] = 1 \cdot 10^{-4}$M.

** - method 2, [complex] = 100 μg/ml, $[AH_2]$ = 227 μg/ml

Determination of antitumor activity of the drugs on mice with regrafted tumors (in vivo)

Tests were carried out on tumor strains: Ehrlich ascitic carcinoma (example 1), ascitic hepatoma 22 (examples 2,

3), solid breast adenocarcinoma Ca-755 (example 4).

The complexes are dissolved in a sterile physiological solution until a concentration of from 0.005 to 1% is obtained. Ascorbic acid is dissolved in sterile distilled water or an isotonic solution of sodium chloride to a concentration of from 0.011 to 2.2%. The complexes and ascorbic acid are administered intraperitoneally, intrapleurally, intravenously or into the tumor itself.

Example 1.

A tumor, an Ehrlich ascitic carcinoma, was grafted into mice intrapleurally. The tests were carried out in a manner similar to that of Example 2.

Mice of the control group without treatment died on the 9th-15th day with development of tumorous pleurisy.

Mice, who had received treatment with a complex of $Co[Pc(COONa)_8]$ in a single dose of 75 mg/kg with subsequent administration of 165 mg/kg of ascorbic acid, lived for 18-40 days. Death from toxicity was not observed (Table 3).

Example 2.

A tumor, ascitic hepatoma 22, was grafted intraperitoneally, the grafting dose was 10^6 cells per mouse. Mice of both sexes were used. The weight of each mouse was at least 18 g. Treatment was begun 48 hours after the tumor was grafted. Mice of the control group without treatment lived 19.3+1.7 days and died with expressed ascites. In the group of mice receiving treatment with a complex of $Co[Pc(COONa)_8]$ in a single dose of 100 mg/kg with subsequent administration of ascorbic acid (a course dose of 550 mg/kg), one mouse died on the 19th day with ascites, the remaining 8 of the 9 mice lived without symptoms of tumor for more than 70 days. Death from toxicity was not observed (Table 3).

Example 3.

Tests were carried out in a manner similar to that in example 1, but the tumor, ascitic hepatoma 22, was grafted to the mice intrapleurally.

Mice of the control group without treatment lived 5.7+1.6 days and died with exudation in the pleural cavity in a volume of about 2.0 ml.

In the group of mice who received treatment with a complex of $Co[Pc(COONa)_8]$ in a single dose of 75 mg/kg with subsequent administration of 165 mg/kg of ascorbic acid, the mice lived more than 70 days without symptoms of a tumor. Death from toxicity was not observed (Table 3).

Table 3

Effect of complex $Co[Pc(COONa)_8]$ and ascorbic acid (AH_2) on the life-span of mice with grafted tumors, as compared with the prototype

Substance	Tumor strain			
	Ehrlich carcinoma		Hepatoma 22	
	ALS, %	Recovery, %	ALS, %	Recovery, %
$Co[Pc(COONa)_8]+AH_2$	296	0	370	70
[Cu(GGH)]Cl(prototype [5])	60	0		

Example 4.

Breast adenocarcinoma Ca-755 was grafted into the mice using 50 mg of tumorous tissue. Treatment was begun 48 hours or on the 9th day after the tumor was grafted. The complexes and ascorbic acid were administered in several ways: intravenously, intraperitoneally, intratumorously (Table 4).

The results obtained with mice having tumors were evaluated by means of generally accepted indexes of antitumor activity, with mice who were not given antitumor therapy being used for control.

Calculation of the increase of life-span was made using the equation:

$$ALS = \frac{L_{test} - L_{control}}{L_{control}} \times 100\%,$$

where L is the life-span in days.

Inhibition of tumor growth was calculated for solid tumors using the equation:

$$TGI = \frac{V_{average\ control} - V_{average\ test}}{V_{average\ control}} \times 100\%,$$

where $V_{average}$ is the average volume of the tumor, calculated as the product of three measurements and expressed in cubic cm.

Regression of the tumor was determined for a developed solid adenocarcinoma Ca-755, the percentage of regression was calculated by the equation:

$$R = \frac{V_o - V_n}{V_o} \times 100\%,$$

where

R is the percentage of regression,

V_o is the initial average volume of a tumor,

V_n is the average volume of a tumor after treatment for "n" days.

Table 4

Inhibition of the growth of a solid breast adenocarcinoma Ca-755 after administration of phthalocyanine complexes of cobalt and ascorbic acid (AH_2)		
Complex	Dose (mg/kg)	TGI*,%
$Co[Pc(CH_2C_5H_4N^{\oplus})_6]Cl^{\ominus}_6$	25	58
AH_2	27.5	38
$Co[Pc(CH_2C_5H_4N^{\oplus})_6]Cl^{\ominus}_6 + AH_2$	25 + 27.5	91
$Co[Pc(SO_3Na)_2]$	25	68
AH_2	56.75	66
$Co[Pc(SO_3Na)_2] + AH_2$	25 + 56.75	88
$Co[Pc(COONa)_8]$	10	44
AH_2	22	38
$Co[Pc(COONa)_8] + AH_2$	10 + 22	61

* - data in respect of Co complexes; $Co[Pc(CH_2C_5H_4N^{\oplus})_6]Cl^{\ominus}_6$ and $Co[Pc(SO_3Na)_2]$ are presented for the 14th day, data in respect of the complex $Co[Pc(COONa)_8]$ are presented for the 16th day after transplantation of the tumors.

Thus, use of the proposed drug makes it possible to suppress effectively the growth of a wide range of malignant tumors, in particular to achieve the suppression of proliferation of cancer cells (in vitro) and inhibition of the growth of tumors in mice (in vivo) and substantially increase their life-span, including that obtained in comparison by use of the prototype.

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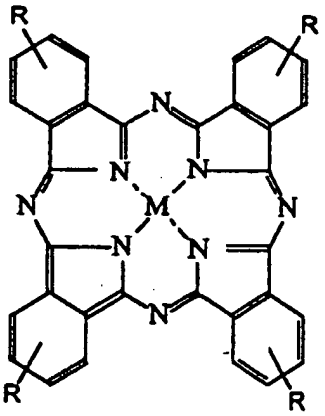
Claims

1. Drug for tumor growth suppression, comprising a complex of a transition metal with a polydentate ligand and a reductant, characterized in that it comprises a complex of cobalt or iron with substituted phthalocyanines or naphthalocyanines of the structural formula I or II:



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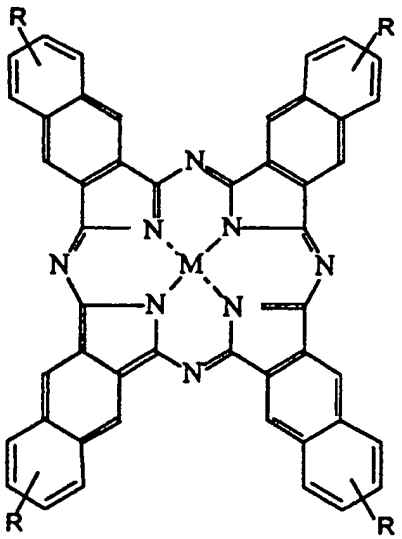

II

where M = Co or Fe, R = COONa, SO_3Na, $CH_2C_5H_4N^{\oplus}Cl^{\ominus}$, $CH_2(NH_2)_2SCH_2Cl$ and a biogenic reductant. with a ratio of the components from 1:5 to 1:50.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/RU 96/00060

A. CLASSIFICATION OF SUBJECT MATTER
IPC[6] A61K 31/40

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC[6] A61K 27/00,31/40,C09B 47/04, C07D 487/22

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO, A, 95/05818 (THE SECRETARY OF STATE FOR DEFENCE), 02 March 1995 (02.03.95), the abstract	1
A	US, A, 5358940 (CIBA-GEIGY CORPORATIJN), 25 October 1994 (25.10.94), the abstract	1
A	US, A, 4393071 (NAOHARU FUJII), 12 July 1983 (12.07.83)	1
A	EP, A1, 0484027 (IMPERIAL CHEMICAL INDUSTRIES PLC), 06 May 1992 (06.05.92), the description, page 8, claims nos. 12-13	

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06 June 1996 (06.06.96)	11 June 1996 (11.06.96)
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